The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

EPO - DG 1

05. 07. 2004

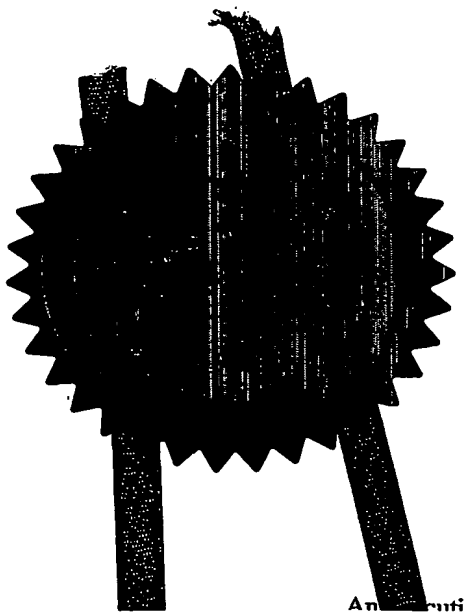
(82)

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.



Signed Andrew Gerry

Dated 6 May 2004

Patents Form 1/77

Patents Act 1977
(Rule 16)

THE PATENT OFFICE
LJ
- 8 MAY 2003
RECEIVED BY FAX

The Patent Office

1/77

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
South Wales
NP9 1RH

1. Your reference
LRD-GB-1-447

08MAY03 E805806-1 D10059
P01/7700 0.00-0310593.9

2. Patent application number
(The Patent Office will fill in this part)
0 8 MAY 2003

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*
K.U.Leuven Research and Development - Groot Begijnhof 59 - 3000 Leuven
0310593.9
Represented by Dr. Patrick Chaltin, T.T.O.
Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation
Belgium
76656 49003

4. Title of the invention
Peptidic prodrugs

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*
K.U.Leuven R&D
care off:
Hubert Velge
Neaves Cottage
Neaves Lane - Glyndebourne
East Sussex BN8 5UA

Patents ADP number *(if you know it)*
7007916003

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))
Yes

Patents Form 1/77

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	29
Claim(s)	1
Abstract	1
Drawing(s)	7 only

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	/
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	2
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	1 fax cover sheet / 1 fee sheet with request for fax back service /

11. I/We request the grant of a patent on the basis of this application.

Dr. Patrick Chaltin — Signature — Date 08/05/2003

12. Name and daytime telephone number of person to contact in the United Kingdom: Hubert Velge +44 7940 540 397

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) *If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.*

b) *Write your answers in capital letters using black ink or you may type them.*

c) *If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.*

d) *If you have answered 'Yes' Patents Form 7/77 will need to be filed.*

e) *Once you have filled in the form you must remember to sign and date it.*

f) *For details of the fee and ways to pay please contact the Patent Office.*

Peptidic prodrugs

FIELD OF THE INVENTION

The invention provides a novel prodrug technology that can be applied to ameliorate the solubility and/or the bioavailability of therapeutic agents. The invention comprises the derivatisation of therapeutic agents in order to ameliorate their solubility and bioavailability. The invention provides conjugates of therapeutic agents with a peptidic moeity wherein said conjugate is cleavable by a dipeptidyl-peptidase. This technology can furthermore be used to modulayte the protein binding of a therapeutic drug and to target specific sites in a mammal.

BACKGROUND OF THE INVENTION

Modern drug discovery techniques (e.g. combinatorial chemistry, high-throughput pharmacological screening, structure based drug design) are providing very specific and potent drug molecules. However, it is rather common that these novel chemical structures have unfavorable physicochemical and biopharmaceutical properties. Besides, during the development of new therapeutic agents, researchers typically focus on pharmacological and/or biological properties, with less concern for physicochemical properties. However, the physicochemical properties (dissociation constant, solubility, partition coefficient, stability) of a drug molecule have a significant effect on its pharmaceutical and biopharmaceutical behavior. Thus, the physicochemical properties need to be determined and modified, if needed, during drug development. Moreover, the physicochemical properties of many existing drug molecules already on the market are not optimal.

Today, drug candidates are often discontinued due to issues of poor water solubility or inadequate absorption, leaving countless medical advances unrealized. Still other products make it to the market, but never realize their full commercial potential due to safety or efficacy concerns. Prodrugs have the potential to overcome both challenges. The technology exploits endogenous enzymes for selective bioconversion of the prodrug to the active form of the drug. This technology has the

ability to keep promising new drug candidates alive through development, and improving the safety and efficacy of existing drug products.

Prodrugs are mostly inactive derivatives of a drug molecule that require a chemical or enzymatic biotransformation in order to release the active parent drug in the body. Prodrugs are designed to overcome an undesirable property of a drug. As such this technology can be applied to improve the physicochemical, biopharmaceutical and/or pharmacokinetical properties of various drugs. Usually, the prodrug as such is biologically inactive. Therefore, prodrugs need to be efficiently converted to the parent drugs to reach pronounced efficacy as soon as the drug target has been reached.

In general, prodrugs are designed to improve the penetration of a drug across biological membranes in order to obtain improved drug absorption, to prolong duration of action of a drug (slow release of the parent drug from a prodrug, decreased first-pass metabolism of the drug), to target the drug action (e.g. brain or tumor targeting), to improve aqueous solubility and stability of a drug (i.v. preparations, eyedrops, etc.), to improve topical drug delivery (e.g. dermal and ocular drug delivery), to improve the chemical / enzymatic stability of a drug (e.g. peptides) or to decrease drug side-effects.

Many prodrug technologies have already been developed depending on the kind of drug that has to be converted. These prodrug technologies include cyclic prodrug chemistry for peptides and peptidomimetics, phosphonooxymethyl (POM) chemistry for the solubilization of tertiary amines, phenols and hindered alcohols and esterification in general. Also targeting strategies are pursued by coupling groups cleavable by specific enzymes such as the peptide deformylase of bacteria which cleaves N-terminal formyl groups of the peptides or PSA (prostate specific antigen) used to target prostate cancer.

Coupling of peptides or amino acids to a therapeutic agent has already been pursued in the past for several reasons. In the antisense-antigene field, oligonucleotides or intercalators have been conjugated to peptides in order to increase the cellular uptake of the therapeutic agents. These oligonucleotides and intercalators have not to be released after cell penetration however, and can not be regarded as prodrugs. An example of amino acid coupling to a therapeutic drug is Valgancyclovir,

the L-valyl ester prodrug of gancyclovir, which is used for the prevention and treatment of cytomegalovirus infections. After oral administration, the prodrug is rapidly converted to gancyclovir by intestinal and hepatic esterases. Recently, alanine and lysine prodrugs of novel antitumor benzothiazoles have been investigated.

Peptide carrier-mediated membrane transport of amino acid ester prodrugs of nucleoside analogues has already been demonstrated (1,2). It has indeed been shown that oral bioavailability of drugs can be mediated by amino acid prodrug derivatives containing an amino acid, preferably in the L-configuration. L-Valine seems to have the optimal combination of chain length and branching at the β-carbon of the amino acid for intestinal absorption. hPEPT-1 has been found to be implicated as the primary absorption pathway of increased systemic delivery of L-valine ester prodrugs. Recently, it was shown that the hPEPT-1 transporter need to optimally interact with a free NH_2, a carbonyl group and a lipophylic entity, and may form a few additional H-bridges with its target molecule. L-Valine-linked nucleoside analogue esters may fulfill these requirements for efficient hPEPT-1 substrate activity (3). The prior art for ameliorating solubility and bioavailability reveals however only amino acid prodrugs (only one amino acid coupled) of small organic molecules whereby the amino acid is mostly coupled through ester bonds, since they are easily converted back to the free therapeutic agent by esterases.

There is however still a need for new and better prodrug technologies and this need is projected to grow, as combinatorial chemistry and high throughput screening continue to produce vast numbers of new compounds with a high molecular weight, high log P, and poor water solubility.

In the present invention, a new prodrug technology is provided based on the coupling of a peptide to a therapeutic agent, whereby the conjugates are cleavable by a dipeptidyl-peptidase. As such, the solubility and bioavailability of the therapeutic drug can be modulated more extensively. The lymphocyte surface glycoprotein CD26 belongs to a unique class of membrane-associated peptidases. It is characterised by an array of diverse functional properties and it is identical to dipeptidyl-peptidase IV (DPP IV, EC 3.4.14.5). DPP IV is a member of the prolyl oligopeptidase (POP; EC3.4.21.26) family, a group of atypical serine proteinases able to hydrolyse the prolyl bond. The 766-amino acid long CD26 is anchored to the cellular lipid bilayer

membrane by a single hydrophobic segment, and has a short cytoplasmic tail of six amino acids (4). The membrane anchor is linked to a large extracellular glycosylated region, a cysteine-rich region and a C-terminal catalytic domain (4). CD26 is strongly expressed on epithelial cells (i.e. kidney proximal tubules, intestine) and on several types of endothelial cells and fibroblasts, as well as leukocyte subsets (5). CD26 also occurs as a soluble form present in seminal fluids, plasma and cerebrospinal fluid. It lacks the intracellular tail and the transmembrane region (6). In addition to its exopeptidase activity, CD26 specifically binds to several proteins outside its substrate-binding site [i.e. adenosine deaminase (7), fibronectin (8), collagen (9)]. CD26 is endowed with an interesting (dipeptidyl) peptidase catalytic activity and it has a high selectivity for peptides with a proline or alanine at the penultimate position of the N-terminus of a variety of natural peptides.

Several cytokines, hematopoietic growth factors, neuropeptides and hormones share the X-Pro or X-Ala motif at their N-terminus and have been shown to act as efficient substrates for the enzyme (for an overview, see references 6a and 6b). Substance P is even an example of a natural peptide of 11 amino acids containing an Arg-Pro-Lys-Pro sequence at its NH_2-terminus, and which is cleaved by CD26 to an active heptapeptide by stepwise release of Arg-Pro and Lys-Pro (10). CD26 can cut dipeptides from very small natural peptides [i.e. the pentapeptide enterostatin (Val-Pro-Asp-Pro-Arg) (11)] to larger peptides [including the chemokines RANTES and SDF-1α and IP-10 (68 to 77 amino acids)] containing respectively the Ser-Pro, Lys-Pro and Val-Pro sequences at their amino terminus (12-15).

Although a relatively restricted substrate specificity (penultimate Pro or Ala) has been observed for CD26, lower cleavage rates have also sometimes been observed when the penultimate amino acids were Gly, Ser, Val or Leu instead of Pro or Ala (6). Also, the nature of the terminal amino acid plays a role in the eventual catalytic efficiency of CD26. There is a decreasing preference from hydrophobic (i.e. Val, Tyr) to basic (i.e. Lys, Arg, His) to neutral (i.e. Gly, Ser, Gln) to acidic (i.e. Asp) amino acids as the preferred first amino acid at the amino terminus for efficient cutting of the peptide by CD26 (6). The observation that a double truncation of macrophage-derived chemokine (MDC) by CD26 can occur thereby sequentially loosing Gly^1-Pro^2 followed by Tyr^3-Gly^4, suggests that the substrate activity of CD26 may be less restricted to the penultimate Pro or Ala than generally accepted (16).

Many other hydrolases (EC 3), more specifically peptidases (EC 3.4) and yet more specifically aminopeptidases (EC 3.4.11) such as prolyl aminopeptidase (EC 3.4.11.5) and X-Pro aminopeptidase (EC 3.4.11.9) have already been identified. Also other dipeptidases (EC 3.4.13), peptidyl-dipeptidases (EC 3.4.15) and dipeptidyl-peptidases (EC 3.4.14, this EC-group also includes tripeptidyl-peptidases) exist next to CD26. Dipeptidyl-peptidase I (EC 3.4.14.1), II (EC 3.4.14.2), III (EC 3.4.14.4), IV (EC 3.4.14.5), dipeptidyl-dipeptidase (EC 3.4.14.6) and X-Pro dipeptidyl-peptidase (EC 3.4.14.11) for example, have already been identified and characterised. Some of them are found in humans and other mammals, while others are produced by micro-organisms such as yeast and fungi. They differ in first instance in amino acid sequence, but also in their specificity for recognising amino acid sequences.

The present invention demonstrates that peptidyl prodrug derivatives are efficiently converted to the parent compound by the exodipeptidyl-peptidase activity of CD26. Since an L-valine moiety can be involved in the dipeptidyl prodrug approach, this technology may represent a powerful tool to make lipohilic compounds not only markedly more water-soluble and less protein binding, but also to enhance oral bioavailability and plasma delivery of the parent molecule and a more selective delivery of the parent drug to CD26-expressing cells.

SUMMARY OF THE INVENTION

The present invention provides a new prodrug technology and new prodrugs in order to modulate the solubility, protein binding and/or the bioavailability of a drug. In the present invention the prodrugs are conjugates of a therapeutic drug and a peptide wherein the conjugate is cleavable by dipeptidyl-peptidases, more preferrably by dipeptidyl-peptidase IV. The present invention furthermore provides a method of producing said prodrugs. The invention also provides a prodrug technology to more selectively target drugs, to enhance brain and lymphatic delivery of drugs and/or to extend drug half-lives in plasma.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. Structural formulae of test compounds.

Fig. 2. Conversion of 50 μM TSAO-NAP-Pro-Val (CAM-405) to TSAO-NAP (CAM-212) by purified CD26 (5.7 mUnits) in function of incubation time (37°C).

Fig. 3. Inhibitory effect of different concentrations of the dipeptide Val-Pro against CD26-catalysed conversion of GP-pNA (25 μM) to GP + pNA at 5, 10 or 15 min of reaction. The CD26 catalytic reaction was measured by recording the increase of absorption caused by pNA release at 400 nm.

Fig. 4. Conversion of 50 μM TSAO-NAP-Pro-Val (CAM-405) to TSAO-NAP (CAM-212) by several dilutions of human serum (HS) (upper panel) and bovine serum (BS) (lower panel) in PBS. Conversion was recorded after 3, 6 or 24 hrs of incubation.

Fig. 5. Inhibitory effect of Diprotin A (panel A) or IlePyr (panel B) on the conversion of 50 μM TSAO-NAP-Pro-Val (CAM-405) to TSAO-NAP (CAM-212) by purified CD26 (1.5 mUnits), 2.5% bovine serum (BS) in PBS or 2.5% human serum (HS) in PBS.

Fig. 6. Conversion of a variety of dipeptide derivatives of TSAO-NAP (50 μM) by 20% human serum in PBS in function of incubation time. Black bars (▦) represent the parent dipeptide derivatives of TSAO-NAP. Dashed bars (▨) represent TSAO-NAP-amino acyl derivatives from which the last amino acid (valine) has been removed. Dotted bars (▩) represent TSAO-NAP (CAM-212) that had been released from the TSAO-NAP-dipeptidyl derivatives.

Fig. 7. Overview of the synthestic scheme used for the synthesis of TSAO derivatives.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "therapeutic drug" as used herein refers to any agent having a beneficial effect on a disease, any agent that is or will be used in the future as a therapy for a certain disease or disorder. This refers also to all molecules which are still in the discovery or development phase and which have not proven their efficacy and safety yet. This includes small organic molecules, proteins, peptides, oligonucleotides and analogs and derivatives.

"CD26" as used herein refers to the dipeptidyl-peptidase IV (EC 3.4.14.5) in its membrane bound and free form.

As used herein, "dipeptidyl-peptidase(s)" refers to enzymes with a dipeptidyl aminopeptidase activity. Other enzymes than CD26 with the same activity and proteolytic specificity as CD26 are referred to by "dipeptidyl-peptidase(s)". By "dipeptidyl-peptidase IV, CD26 is referred to.

The term "prodrug" as used herein refers to derivatives of a therapeutic drug that requires a chemical or enzymatic biotransformation in order to release the active parent therapeutic drug in the body.

As written herein, amino acid sequences are presented according to the standard convention, namely that the amino terminus of the peptide is on the left and the carboxy terminus is on the right.

As used herein, the term "peptide" is considered as of the moment that a molecule comprises two amino acids coupled via an amide bond.

The present invention is based on the observation that the dipeptidyl-peptidase IV (CD26) has a postproline or postalanine dipeptidyl aminopeptidase activity, preferentially cleaving X-proline or X-alanine dipeptides from the N-terminus of polypeptides or proteins.

In view of this observation, the present invention provides a new prodrug technology in order to modulate the solubility, plasma protein binding and/or to enhance the bioavailability of a drug. In other embodiments of the invention, prodrugs are delivered in order to more selectively target drugs, to enhance brain and lymphatic

delivery of drugs and/or to extend drug half-lives in plasma. The present invention provides new prodrugs, characterised in that the prodrugs are cleavable by a dipeptidyl-peptidase or other enzymes with the same activity and proteolytic specificity as CD26. In a preferred embodiment, the prodrugs of the present invention are peptide-drug conjugates and derivatives thereof, that include amino acid sequences containing cleavage sites for dipeptidyl-peptidases. As such, the invention also provides a therapeutic prodrug composition comprising a therapeutic drug linked to a peptide which is specifically cleaved by dipeptidyl-peptidases.

The therapeutic drug can be linked to the carboxy group of an amino acid either directly or through a linker group. In a preferred embodiment, the therapeutic drug and the peptide are directly coupled via an amide bond. The therapeutic drug can have a free amino group (primary or secondary) amide that can be coupled with the carboxyl group of amino acids, more preferrably with the α-carboxyl group. In another preferred embodiment, the therapeutic drug and the peptide are coupled via a linker, wherein the linker can be of non-peptidic or peptidic nature. If the connection between the therapeutic drug and the peptide is made through a linker, the connection between the linker and the first amino acid is preferrably an amide bond. The linker may be connected to the therapeutic drug through any bond types and chemical groups known to those skilled in the art, more preferably by covalent bonding. The linker may remain on the therapeutic drug indefinitely after cleavage, or may be removed thereafter, either by further reactions with external agents or in a self-cleaving step. External agents which may affect cleavage of the linker include enzymes, proteins, organic or inorganic reagents, protons and other agents. In embodiments in which the linker remains attached to the drug, the linker can be any group which does not substantially inhibit the activity of the drug after cleavage of the peptide. In other embodiments, the linker is self-cleaving. Self-cleaving linkers are those which are disposed to cleave from the drug after the cleavage of the peptide by dipeptidyl-peptidases. Mechanisms involved in the self-cleavage of the linkers are for example intra molecular cyclisation or spontaneous S_N1 solvolysis and release the drug upon upon peptide cleavage.Some examples of linkers are provided in Atwell et al. (Atwell et al. *J. Med. Chem.* 1994, 37: 371-380). The linkers generally contain primary amines which form amide bonds to the carboxy terminus of the peptide. The

linkers can also contain a carboxylic acid which forms an amide bond to a primary amine found on the drug. The linker can be coupled to the drug by one or more reactions chosen from the reactions available to the person skilled in the art.

Preferably, the aminoterminal end of the peptide in the prodrug comprises X-Pro, X-Ala, X-Gly, X-Ser, X-Val, or X-Leu, wherein X represents any amino acid or isomers (i.e. L- or D-configuration) thereof. In a preferred embodiment, the peptide comprises aminoterminally X-Proline or X-Alanine. As such the amino acids can be selected from alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine and derivatives thereof. Also modified (i.e. hydroxylproline) or unnatural amino acids can be included. In another preferred embodiment, the length of the peptide is between 2 and 10 amino acids and can therefore have a length of 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids. In another preferred embodiment, the peptide comprises $(X\text{-}Y)_n$ repeated units wherein X represents any amino acid, Y is selected from Pro, Ala, Gly, Ser, Val or Leu and n is selected from 1, 2, 3, 4 or 5. In another more preferred embodiment, said peptide is a dipeptide. In still a more preferred embodiment, the dipeptide is Lys-Pro. In another still more preferred embodiment, the amino acids have the L-configuration. The aminoterminus of the peptide may also contain conventional capping groups. Such capping groups include acetyl, succinyl, benzyloxycarbonyl, glutaryl, morpholinocarbonyl, methyl and many others known in the art. Those skilled in the art can make substitutions to achieve peptides with better profile related to solubility, bioavailability and targeting of the conjugate. Therefore, the invention includes the peptide sequences as described above, as well as analogs or derivatives thereof, as long as the conjugates remain cleavable by dipeptidyl-peptidase.

The therapeutic drugs that may be used in the prodrugs of the invention include any drugs that can be directly or indirectly linked to a peptide and whereby the conjugate is cleavable by dipeptidyl-peptidase. In addition to known therapeutic drugs, this invention can also be applied to the novel drug molecules that are currently under drug development or to drug molecules which are already in clinical use. In another preferred embodiment, the therapeutic drug is a small organic molecule and not a peptide, protein, an intercalator or an oligonucleotide or analogs thereof (such as

HNA, PNA, etc.). The therapeutic molecule can have an activity in the cardiovascular, neurological, respiratory, oncology, metabolic diseases, immunology, urology, anti-infectives, inflammation and all other therapeutic fields. In yet another more preferred embodiment, the therapeutic drug has an antiviral activity. In still a more preferred embodiment, the therapeutic drug has an anti-HIV activity.

Preferred drugs are those containing primary amines. The presence of a primary amine allows the formation of an amide bond between the drug and the peptide. The primary amines may be found in the drugs as commonly provided, or they may be added to the drugs by chemical synthesis. Certain therapeutic drugs contain primary amines, for example, anthracycline antibiotics containing containing an amino sugar such as doxorubicin, daunorubicin, epirubicin, idarubicin and the like. Antiviral drugs that contain an amine or amide are for example the guanine derivatives with anti-herpes activity like acyclovir, gancyclovir, penciclovir and lobucavir, the cytosine derivatives gemcitabine, ddC, araC and lamivudine (3TC), the protease inhibitors amprenavir and DMP850 and 851. Others are ribavirin, the NNRTIs TMC125 (from Tibotec-Virco) and AG1549 (from Agouron), PMPA (tenofovir), PMEA (adefovir) and oseltamivir.

According to the FDA's Biopharmaceutics Classification System (BCS), drug substances are classified as follows: Class I - High Permeability, High Solubility; Class II - High Permeability, Low Solubility; Class III - Low Permeability, High Solubility and Class IV - Low Permeability, Low Solubility. How drugs are classified in this classification system is described in the guidlines of the BCS. In a preferred embodiment, the therapeutic drugs that can be used in the invention are selected from class II, III and IV.

The invention provides for prodrugs that are cleavable by dipeptidyl-peptidases. The dipeptidyl-peptidases can be selected from the group of peptidases (EC 3.4) and yet more specifically aminopeptidases (EC 3.4.11) such as prolyl aminopeptidase (EC 3.4.11.5) and X-Pro aminopeptidase (EC 3.4.11.9), from the group of dipeptidases (EC 3.4.13), peptidyl-dipeptidases (EC 3.4.15) and dipeptidyl-peptidases (EC 3.4.14, this EC-group also includes tripeptidyl-peptidases) such as dipeptidyl-peptidase I (EC 3.4.14.1), II (EC 3.4.14.2), III (EC 3.4.14.4), IV (EC 3.4.14.5), dipeptidyl-dipeptidase (EC 3.4.14.6) and X-Pro dipeptidyl-peptidase (EC 3.4.14.11). In a preferred embodiment, the prodrug is cleavable by dipeptidyl-peptidases present in mammals or

more preferrably in humans. In a more preferred embodiment, the prodrug is cleavable by dipeptidyl-peptidase IV (CD26), as well by the cell-surface bound as by the soluble form present in seminal fluids, plasma and cerebrospinal fluid.

The invention also provides a method for modulating (i.e. increasing) the (water) solubility, the protein binding and/or the bioavailability of a therapeutic drug by coupling a peptide to said therapeutic drug whereby the resulting conjugate is cleavable by a dipeptidyl-peptidase. Any changement of the therapeutic drug, also including conjugation of amino acids, has a proven influence on the solubility and bioavailability profile of said drug. The present invention provides however a method of ameliorating the solubility and/or bioavailability of the drug without changing the activity profile of the therapeutic drug. Other chemical groups may be coupled to the prodrugs of the invention, including those which render the prodrug more soluble in water. These groups include polysaccharides or other polyhydroxylated moieties. For example, dextran, cyclodextrin and starch may be included in the prodrug of the invention.

The present invention also provides a method for targeting molecules to dipeptidyl-peptidase expressing cells, tissues or organs, provided that the dipeptidyl-peptidases are expressed on the cell surface or secreted in the extracellular medium.

The present invention furthermore provides a method of producing a prodrug, wherein the prodrug is cleavable by a dipeptidyl-peptidase. This method of producing a prodrug comprises the step of linking a therapeutically active drug and a peptide. In a more preferred embodiment, the therapeutically active drug or the peptide are in a first step derivatised in order to be able to link the therapeutic drug and the peptide in a later step via an amide bond. In certain embodiments, the peptide is linked directly to the drug. In other embodiments, the peptide is indirectly linked to the drug, the linkage occurring through a linker. In each case the carboxy terminus of the peptide is used for linking. Many acceptable methods of coupling carboxyl and amino groups to form amide bonds are known to those skilled in the art.

The present invention furthermore provides for prodrugs of TSAO. Peptide prodrugs of [1-[2',5'-Bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl]-3-(3-

amino-propyl)-thymine]-(R)(ribo)-3'-spiro-5-(4-amino-1,2-oxathiole-2,2-dioxide) (=TSAO-NAP) are provided by this invention. The valine, valine-proline and valine-proline-valine TSAO-NAP derivatives are provided by this invention.

In another aspect the invention provides a method of detecting dipeptidyl-peptidase producing tissue or cells by using the prodrug technology of the invention, as described above. The method is carried out by contacting a detectably labeled peptide of the invention with target tissue for a period of time sufficient to allow dipeptidyl-peptidase to cleave the peptide and release the detectable label. The detectable label is then detected. The level of detection is then compared to that of a control sample not contacted with the target tissue. Many varieties of detectable label are available, including optically based labels, such as chromophoric, chemiluminescent, fluorescent or phosphorescent labels, and radioactive labels, such as alpha, beta or gamma emitting labels. Examples of fluorescent labels include amine-containing coumarins such as 7-amino-4-methylcoumarin, 7-amino-4-trifluoromethyl, and other amine-containing fluorophores such as 6-aminoquinoline, 2-aminopurines, and rhodamines, including rhodamine 110. Examples of radioactive labels include beta emitters such as ^{3}H, ^{14}C and ^{125}I. Examples of chromophoric labels (those that have characteristic absorption spectra) include nitroaromatic compounds such as p-nitroaniline. Examples of chemiluminescent labels include luciferins such as 6-amino-6-deoxyluciferin.
Preferably, the choice of detectable label allows for rapid detection and easily interpretable determinations. Detectable labels for use in the invention preferably show clearly detectable differences between detection from the cleaved and uncleaved state.

The invention provides a method for detecting a disorder accompanied with overexpression of dipeptidyl-peptidases, more preferrably CD26, which comprises contacting a prodrug with a cell suspected of having a dipeptidyl-peptidase-production associated disorder and detecting cleavage of the peptide. The peptide reactive with dipeptidyl-peptidase is labeled with a compound which allows detection of cleavage by dipeptidyl-peptidase. For purposes of the invention, a prodrug may be used to detect the level of enzymatically active dipeptidyl-peptidase in biological fluids and tissues such as saliva, blood, or urine. The level of dipeptidyl-peptidase in

the suspected cell can be compared with the level in a normal cell to determine whether the subject has a dipeptidyl-peptidase-production associated cell disorder. Preferably the subject is human.

The invention also provides a method of selecting potential prodrugs for use in the invention. The method generally consists of contacting prodrugs of the invention with dipeptidyl-peptidases or tissue or cells producing dipeptidyl-peptidases and with dipeptidyl-peptidases free medium in a parallel experiment.

In a certain embodiment of the invention, the above described prodrugs can be used as a medicine. In another embodiment, the above described prodrugs can be used to manufacture a medicament to prevent or to treat a certain disease. The disease that will be treated depends on the therapeutical drug that will be used in the prodrug technology.

The invention furthermore provides methods of treating a certain disease by administering a prodrug as described by the invention. The prodrugs of the invention and/or analogs or derivatives thereof can be administered to any host, including a human, a non-human animal and mammals, in an amount effective to treat a disorder.

To further optimise the pharmacokinetic profile of the prodrugs of present invention they can be administered in conjunction with a suitable delivery vehicle (e.g., microcapsules, microspheres, biodegradable polymer films, lipid-based delivery systems such as liposomes and lipid foams, viscous instillates and absorbable mechanical barriers) useful for maintaining the necessary concentrations of the prodrugs or the therapeutic drug at the site of the disease.

The prodrug or "medicament" may be administered by any suitable method within the knowledge of the skilled man. Modes of administration known in the art for therapeutic agents include parenteral, for example, intravenous (e.g. for antibody inhibitors), intraperitoneal, intramuscular, intradermal, and epidermal including subcutaneous and intradermal, oral, or application to mucosal surfaces, e.g. by intranasal administration using inhalation of aerosol suspensions, and by implanting to

muscle or other tissue in the subject. Suppositories and topical, locally applied preparations are also contemplated.

In the present invention, the prodrugs are introduced in amounts sufficient to prevent, reduce or treat a certain disease, depending on the administration route.

The most effective mode of administration and dosage regimen for the prodrugs or the "medicament" in the methods of the present invention depend on the severity of the disease to be treated, the subject's health, previous medical history, age, weight, height, sex and response to treatment and the judgment of the treating physician. Therefore, the amount of prodrug to be administered, as well as the number and timing of subsequent administrations are determined by a medical professional conducting therapy based on the response of the individual subject. Initially, such parameters are readily determined by skilled practitioners using appropriate testing in animal models for safety and efficacy, and in human subjects during clinical trials of prodrug formulations. After administration, the efficacy of the therapy using the prodrugs is assessed by various methods including assessment of the clinical picture.

Suitable pharmaceutical carriers for use in said pharmaceutical compositions and their formulation are well known to those skilled in the art, and there is no particular restriction to their selection within the present invention. Suitable carriers or excipients known to the skilled man are saline, Ringer's solution, dextrose solution, Hank's solution, fixed oils, ethyl oleate, 5% dextrose in saline, substances that enhance isotonicity (such as sugars or sodium chloride) and chemical stability, buffers and preservatives. Other suitable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids and amino acid copolymers. They may also include additives such as wetting agents, dispersing agents, stickers, adhesives, emulsifying agents, solvents, coatings, antibacterial and antifungal agents (for example phenol, sorbic acid, chlorobutanol) and the like, provided the same are consistent with pharmaceutical practice, i.e. carriers and additives which do not create permanent damage to mammals. The pharmaceutical compositions of the present invention may be prepared in any known manner, for instance by homogeneously mixing, coating and/or grinding the active ingredients, in a one-step or multi-steps procedure, with the selected carrier material

and, where appropriate, the other additives such as surface-active agents may also be prepared by inicronisation, for instance in view to obtain them in the form of microspheres usually having a diameter of about 1 to 10 μm, namely for the manufacture of microcapsules for controlled or sustained release of the active ingredients.

Suitable surface-active agents to be used in the pharmaceutical compositions of the present invention are non-ionic, cationic and/or anionic materials having good emulsifying, dispersing and/or wetting properties. Suitable anionic surfactants include both water-soluble soaps and water-soluble synthetic surface-active agents. Suitable soaps are alkaline or alkaline-earth metal salts, unsubstituted or substituted ammonium salts of higher fatty acids (CIO-C22), e.g. the sodium or potassium salts of oleic or stearic acid, or of natural fatty acid mixtures obtainable form coconut oil or tallow oil. Synthetic surfactants include sodium or calcium salts of polyacrylic acids; fatty sulphonates and sulphates; sulphonated benzimidazole derivatives and alkylarylsulphonates. Fatty sulphonates or sulphates are usually in the form of alkaline or alkaline-earth metal salts, unsubstituted ammonium salts or ammonium salts substituted with an alkyl or acyl radical having from 8 to 22 carbon atoms, e.g. the sodium or calcium salt of lignosulphonic acid or dodecylsulphonic acid or a mixture of fatty alcohol sulphates obtained from natural fatty acids, alkaline or alkaline-earth metal salts of sulphuric or sulphonic acid esters (such as sodium lauryl sulphate) and sulphonic acids of fatty alcohol/ethylene oxide adducts. Suitable sulphonated benzimidazole derivatives preferably contain 8 to 22 carbon atoms. Examples of alkylarylsulphonates are the sodium, calcium or alcanolamine salts of dodecylbenzene sulphonic acid or dibutyl-naphtalenesulphonic acid or a naphtalene-sulphonic acid/formaldehyde condensation product. Also suitable are the corresponding phosphates, e.g. salts of phosphoric acid ester and an adduct of p-nonylphenol with ethylene and/or propylene oxide, or phospholipids. Suitable phospholipids for this purpose are the natural (originating from animal or plant cells) or synthetic phospholipids of the cephalin or lecithin type such as e.g. phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerine, lysolecithin, cardiolipin, dioctanylphosphatidyl-choline, dipalmitoylphoshatidyl -choline and their mixtures.

Suitable non-ionic surfactants include polyethoxylated and polypropoxylated derivatives of alkylphenols, fatty alcohols, fatty acids, aliphatic amines or amides containing at least 12 carbon atoms in the molecule, alkylarenesulphonates and dialkylsulphosuccinates, such as polyglycol ether derivatives of aliphatic and cycloaliphatic alcohols, saturated and unsaturated fatty acids and alkylphenols, said derivatives preferably containing 3 to 10 glycol ether groups and 8 to 20 carbon atoms in the (aliphatic) hydrocarbon moiety and 6 to 18 carbon atoms in the alkyl moiety of the alkylphenol. Further suitable non-ionic surfactants are water-soluble adducts of polyethylene oxide with poylypropylene glycol, ethylenediaminopolypropylene glycol containing 1 to 10 carbon atoms in the alkyl chain, which adducts contain 20 to 250 ethyleneglycol ether groups and/or 10 to 100 propyleneglycol ether groups. Such compounds usually contain from I to 5 ethyleneglycol units per propyleneglycol unit. Representative examples of non-ionic surfactants are nonylphenol - polyethoxyethanol, castor oil polyglycolic ethers, polypropylene/ polyethylene oxide adducts, tributylphenoxypolyethoxyethanol, polyethyleneglycol and octylphenoxypolyethoxyethanol. Fatty acid esters of polyethylene sorbitan (such as polyoxyethylene sorbitan trioleate), glycerol, sorbitan, sucrose and pentaerythritol are also suitable non-ionic surfactants.

Suitable cationic surfactants include quaternary ammonium salts, preferably halides, having 4 hydrocarbon radicals optionally substituted with halo, phenyl, substituted phenyl or hydroxy; for instance quaternary ammonium salts containing as N-substituent at least one C8C22 alkyl radical (e.g. cetyl, lauryl, palmityl, myristyl, oleyl and the like) and, as further substituents, unsubstituted or halogenated lower alkyl, benzyl and/or hydroxy-lower alkyl radicals.

A more detailed description of surface-active agents suitable for this purpose may be found for instance in "McCutcheon's Detergents and Emulsifiers Annual" (MC Publishing Crop., Ridgewood, New Jersey, 1981), "Tensid-Taschenbuch', 2 d ed. (Hanser Verlag, Vienna, 1981) and "Encyclopaedia of Surfactants, (Chemical Publishing Co., New York, 1981).

Additional ingredients may be included in order to control the duration of action of the active ingredient in the composition. Control release compositions may thus be achieved by selecting appropriate polymer carriers such as for example polyesters, polyamino acids, polyvinyl pyrrolidone, ethylene-vinyl acetate copolymers, methylcellulose, carboxymethylcellulose, protamine sulfate and the like. The rate of

drug release and duration of action may also be controlled by incorporating the active ingredient into particles, e.g. microcapsules, of a polymeric substance such as hydrogels, polylactic acid, hydroxymethylcellulose, polyniethyl methacrylate and the other above-described polymers.

Such methods include colloid drug delivery systems like liposomes, microspheres, microemulsions, nanoparticles, nanocapsules and so on. Depending on the route of administration, the pharmaceutical composition may require protective coatings. Pharmaceutical forms suitable for injectionable use include sterile aqueous solutions or non-aqueous solutions or dispersions (suspensions, emulsions) and sterile powders for the extemporaneous preparation thereof. Typical carriers for this purpose therefore include biocompatible aqueous buffers, ethanol, glycerol, propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate and the like and mixtures thereof. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer, s dextrose), and the like. Preservatives and other additives can also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases, and the like.

The present invention thus provides in a preferred embodiment di- and oligopeptidyl derivatives of drugs that act as efficient substrates for dipeptidyl-peptidases present on the surface of cells or in plasma. By linking for example water-insoluble, lipophilic drugs to (polar) di- or oligopeptides, these drugs become more water-soluble in biological fluids and physiological media, but may also gain (oral) bioavailability due to specific recognition by the intestinal hPEPT-1 and related peptide transporters. Valine derivatives of nucleoside analogues such as valacyclovir and valganciclovir are examples of nucleoside prodrugs that are substrate for hPEPT-1, and whose solubility, absorption and systemic availability has been markedly improved compared with the parent compounds due to intestinal epithelial brush-border membrane peptide-carrier-mediated transport. Modifying the number and nature of the amino acids in the (oligo)peptide part influences the dipeptidyl-peptidase (i.e. CD26) susceptibility of the prodrug molecule, but also the degree of aqueous solubility, plasma protein binding and bioavailability, as well as plasma half-life. The

amino acid composition can be optimized in function of the nature and biological application of the particular drug.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

Examples

Conversion of TSAO-NAP-Pro-Val and TSAO-NAP-Val-Pro-Val to the parent compounds TSAO-NAP and TSAO-NAP-Val by purified CD26

The lipophylic N^3-aminopropyl-substituted TSAO-m^3T nucleoside derivative TSAO-NAP (CAM-212) has been chemically linked to the dipeptide Val-Pro and the tripeptide Val-Pro-Val through the free carboxylic acid end of the di/tripeptide resulting in CAM-405 (TSAO-NAP-Pro-Val) and CAM-431 (TSAO-NAP-Val-Pro-Val) (Fig. 1). To reveal whether CD26 recognizes such synthetic di/tripeptide derivatives of lipophylic nucleoside analogues as a substrate, 50 µM CAM-405 or CAM-431 was exposed to 5.7 mUnits of purified CD26, and the conversion of CAM-405 or CAM-431 to CAM-212 was recorded in function of incubation time by HPLC analysis. The identity of the formed CAM-212 product was revealed by HPLC analysis using the reference parent compound as control, and by mass spectrometry. We found that CD26 efficiently removed the dipeptide Val-Pro from CAM-405 resulting in the time-dependent appearance of the parent compound CAM-212 (Fig. 2). Within the first 10 min of the reaction, at least 20% of CAM-405 had been converted to CAM-212, fifty percent of the TSAO dipeptide derivative was converted to CAM-212 within 30 min of the reaction and ~ 67% of CAM-405 was hydrolysed after 60 min. Thus, the reaction rate started already to slow down after the first 10 min of drug exposure (Fig. 2). Most likely, the release of the reaction product Val-Pro dose-dependently feed-back inhibited the CD26-catalysed dipeptidyl-peptidase reaction. Similar data were obtained for the conversion of the tripeptide derivative TSAO-NAP-Val-Pro-Val (CAM-411) to the TSAO-NAP-Val product (CAM-403) (data not shown). When the dipeptide Val-Pro was evaluated for its inhibitory effect against CD26, 4 mM completely inhibited the reaction; 400 µM Val-Pro inhibited the reaction by >> 90%, whereas 40 µM and 4 µM prevented CD26-catalysed p-

nitroaniline release from GP-pNA by 70 and 15%, respectively (Fig. 3). Thus, in the presence of 50 µM CAM-405, hydrolysis of 50% of CAM-405 to CAM-212 results in the appearance of 25 µM Val-Pro, that is a concentration that (feed-back) inhibits the CD26 reaction by ~ 50%. These findings explain why the CD26-catalysed reaction levels-off shortly after the start of the exposure of the drug to CD26. In contrast, the dipeptide Lys-Pro could be completely removed from TSAO-NAP-Pro-Lys by CD26, pointing to a lack of feed-back inhibition of CD26 by free Lys-Pro.

Conversion of TSAO-NAP-Pro-Val and TSAO-NAP-Val-Pro-Val to the parent compounds TSAO-NAP and TSAO-NAP-Val by human and bovine serum

Human and bovine serum were incubated for 3 hr, 6 hr and/or 24 hr at 37°C in the presence of 50 µM CAM-405. The sera were diluted in PBS at a final concentration of 0.5, 1, 2.5 or 5%. Both human (HS) and bovine (BS) serum efficiently converted CAM-405 to CAM-212. The longer the incubation time, and the higher the serum concentration used, the faster the conversion of CAM-405 to CAM-212 occurred (Fig. 4). As also noted for CD26, HS- and BS-catalysed reaction slowed down in function of time, and was not linearly proportional with serum concentration (Fig. 4). These findings provide again evidence for a pronounced feed-back inhibition of dipeptidyl-peptidase activity in human and bovine serum by the released Val-Pro dipeptide. HS was more efficient in converting CAM-405 to CAM-212 than BS (Fig. 4). Since 1% HS is able to hydrolyse ~ 20% of 50 µM CAM-405 within 3 hrs of incubation, it could be calculated that undiluted serum would have been able to convert this prodrug amount to its parent compound at a hundred fold higher speed, that is, within 1.8 min, provided that no feed-back inhibition would have occurred (as expected in the intact organism where release of Pro-Val would immediately result in disappearance from the plasma due to several mechanisms including organ uptake, renal excretion, etc.). This means that 10 µM CAM-405 should have a half-life of less than 1.8 min in plasma, and thus, will virtually immediately be converted to its parental drug as soon as it appears in the plasma.

Conversion of TSAO-NAP-Pro-Val to the parent compound TSAO-NAP by CEM cell suspensions

The conversion of TSAO-NAP-Pro-Val (CAM-405) to TSAO-NAP (CAM-212) also efficiently occurred by carefully washed T-lymphocytic CEM cell suspensions in PBS. Ten million CEM cells suspended in 200 µl PBS hydrolysed the Val-Pro moiety from CAM-405 by 65% within 3 hrs of incubation at 37°C. This amount of hydrolysis was found both in the PBS supernatant and in the CEM cell extracts. Presumably, CD26 present in the cell membrane of CEM cells had cleaved-off the Val-Pro from CAM-405 after which both truncated and intact prodrug had been taken up by the lymphocytic cells to an equal extent.

Effect of specific CD26 inhibitors on the conversion of TSAO-NAP-Pro-Val to TSAO-NAP

CD26-catalysed CAM-405 conversion to CAM-212 was recorded in the absence or presence of the CD26 inhibitors diprotin A and IlePyr (Fig. 5). Interestingly, at the highest concentration of the inhibitors (500-1000 µM), a nearly complete prevention of the conversion of CAM-405 to CAM-212 occurred in both HS and BS or by purified CD26. At 10-fold lower inhibitory concentrations (i.e. 50-100 µM) diprotin A and IlePyr still efficiently suppressed (>> 50%) the CD26-catalysed conversion of CAM-405 to CAM-212 by purified CD26 preparations and by HS and BS (Fig. 5). These observations point to CD26 as the main and predominant enzyme responsible in HS and BS to remove the dipeptide part from the lipophylic TSAO-NAP dipeptide nucleoside analogue.

Hydrolysis of dipeptide prodrugs in the presence of purified CD26 and human serum

A variety of different TSAO-NAP dipeptide and tripeptide derivatives were synthesized and evaluated for their ability to act as an efficient substrate for CD26. CAM-431 (TSAO-NAP-Val-Pro-Val), containing a tripeptide (Val-Pro-Val) moiety linked to TSAO-NAP was also hydrolysed by CD26, releasing the dipeptide Val-Pro and the remaining valine-substituted TSAO-NAP-Val. Interestingly, CAM-407 (TSAO-NAP-Val-Pro-Val-CH_3) containing a methyl group at the free amino group of Val in CAM-412 completely lacked substrate activity for CD26. Even after 24 hrs of incubation, no traces of a formed truncated TSAO-NAP-Val-Pro-Val-CH_3 derivative could be observed. Similar observations were made for TSAO-NAP-Pro-Val or

TSAO-NAP-Val-Pro-Val derivatives at which a lipophylic entity was linked on the free amino group of valine. Thus, a free amino group on the ultimate amino acid is a prerequisite for substrate activity by CD26. In addition to Val-Pro, we also found Lys-Pro a very efficient dipeptide to be cleaved by CD26. Asp-Pro was much less efficiently cleaved.

When the dipeptide Val-Pro on TSAO-NAP was replaced by other dipeptides such as Val-Gly, Val-Leu or Val-Phe, no CD26-catalysed conversion to the parent compound was observed, even after 24 hrs of incubation. Also, when L-Pro in TSAO-NAP-Pro-Val was replaced by D-Pro, the compound did not act anymore as a measurable substrate for purified CD26, and Val-(D)Pro was not split-off. However, Val-Ala linked to TSAO-NAP, was the only alternative dipeptide found, together with Val-Pro, that was released from the parent TSAO-NAP molecule by CD26. Thus, as with natural peptides that contain a penultimate Pro or Ala at their NH2 terminal, CD26 is also able to recognize this dipeptide sequence when linked through an amide bond to a molecule (i.e. TSAO) different from a peptide.

Interestingly, when the TSAO-NAP-dipeptides were exposed to 20% human serum (diluted in PBS), the compounds were converted to one or two derivatives depending the nature of the dipeptide (Fig. 6). For example, TSAO-NAP-Gly-Val was efficiently (but solely) converted to TSAO-NAP-Gly. TSAO-NAP-Leu-Val and TSAO-NAP-Phe-Val did convert to a limited extent to TSAO-NAP-Leu and TSAO-NAP-Phe, respectively, but also to TSAO-NAP. Interestingly, the TSAO-NAP-D-Pro-Val derivative that contains a penultimate proline residue in D-configuration, is very stable in the human serum. Only a very limited amount of TSAO-NAP (but not TSAO-NAP-D-Pro) had been detected (Fig. 6). The tripeptide derivative TSAO-NAP-Val-Pro-Val was very efficiently converted predominantly to TSAO-NAP-Val by human serum as also occurred in the presence of purified CD26 (data not shown).

Solubility

Lipophilicity of a drug may strongly determine its solubility, plasma protein binding but also its ability to cross the blood-brain barrier. Different dipeptides or tetrapeptides linked to TSAO-NAP markedly influence the calculated log P values of the molecules (Table 1).

It is also clear that the nature of the dipeptide moiety present on TSAO-NAP markedly affect prodrug solubility in water. For example, only little amounts of

prodrug appears in the water phase when Val-Ala had been linked to TSAO-NAP, whereas Val-Gly and particularly Pro-Val linked to TSAO-NAP, had markedly increased water solubility (Table 2)

TABLE 1. CALCULATED LOG P VALUES OF TEST COMPOUNDS

Compound	Log Pa
1. TSAO-m3T	3.21
2. TSAO-NAP	2.38
3. TSAO-NAP-Pro-Val	3.08
4. TSAO-NAP-OH-Pro-Val	2.19
5. TSAO-NAP-Ala-Val	2.41
6. TSAO-NAP-Pro-Ser	1.25
7. TSAO-NAP-Pro-Lys	1.85
8. TSAO-NAP-Pro-Asp	-0.59
9. TSAO-NAP-Pro-Asn	1.00
10. TSAO-NAP-Pro-Lys-Pro-Val	2.93
11. TSAO-NAP-Pro-Asp-Pro-Val	0.64
12. TSAO-NAP-Pro-Val-Pro-Val	4.15

TABLE 2. SOLUBILITY OF TEST COMPOUNDS AFTER 2 X 10 SEC SONICATION AND 4 DAYS SHAKING OF 1 MG/ML COMPOUND IN MILLI-Q WATER AT ROOM TEMPERATURE[A]

Compound	Solubility (HPLC)	Spectrum (~ 265 nm)	R_t (min)
CAM-422 (TSAO-NAP-Gly-Val)	1,120,567	1.886	28.5
CAM-424 (TSAO-NAP-Ala-Val)	229,432	0.650	28.8
CAM-430 (TSAO-NAP-D-Pro-Val)	6,174,671	3.220	31.4
TSAO-m^3T	0	0.100	"22.6"

[a]After shaking: centrifugation 50 min 15,000 rpm → U.V. spectrum or filter (0.45 μ) → quantification by HPLC analysis (acetonitrile/Na phosphate buffer + heptanesulfonic acid).

As a conclusion, dipeptidyl or tripeptidyl derivatives of the lipophylic TSAO nucleoside analogue were shown to be efficient substrates for purified CD26, as well as for soluble CD26 activity present in human and bovine serum. Oligopeptide derivatives of highly lipophylic water-insoluble drugs can make these drugs markedly more water-soluble, less plasma protein binding and can also increase their oral bioavailability. In addition, this technology allows a more specific targeting of drugs to CD26-expressing cells.

Materials and Methods

Compounds, enzymes and cells

The TSAO derivatives depicted in Fig. 1 can be synthesised as described below. GlyPro-pNA (GP-pNA), Diprotin A and Val-Pro were purchased from Sigma-Aldrich (Bornem, Belgium). L-isoleucine pyrrolidine (IlePyr) was synthesized as described (17). CD26 was purified as described before (18). Foetal bovine serum (FBS) was obtained from Integro (Dieren, The Netherlands). Human serum represented a pooled serum that was derived from 10 healthy volunteers (blood donors). Human lymphocyte CEM cells were derived from the ATCC (Rockville, MD).

Synthesis of the TSAO derivatives

N-benzoyloxycarbonyl-3-bromo-propylamine (1)

To an ice cooled suspension of 3-bromopropylamine bromhydrate (0.9 g, 4.11 mmol) and triethylamine (1.3 mL, 9.05 mmol) in dry dichloromethane was slowly added a solution of benzyl chloroformiate (0.6 mL, 4.11 mmol) in dry dichloromethane (1 mL). The reaction mixture was stirred at room temperature overnight. Then it was washed with saturated aqueous NaCl (2 x 15 mL), dryed (anhidrous Na_2SO_4), filtered and evaporated to dryness. The residue was purified by CCTLC on the chromatotron using hexane, ethyl acetate (4:1), to give 0.8 g (72 %) of (1) as a white foam.

[1-[2',5'-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-(3-(benzyl oxicarbonylmetyl)aminopropyl)thymine]-3'-spiro-5''-(4''-amino-1'',2''-oxathiole 2'',2''-dioxide) (2)

To a solution of TSAO-T (1 equiv.) in dry acetone (20 mL) was added dry K_2CO_3 (1.1 equiv.) and compound 1 (2 equiv.). The reaction mixture was refluxed for 6 h, and then, concentrated to dryness. The residue was dissolved in ethyl acetate (20 mL), washed with brine (2 x 20 mL), dried (Na_2SO_4), filtered and evaporated to dryness. The residue thus obtained was purified by flash column chromatography, using dichloromethane:metanol (70:1) as the eluent to give 2 (85 %) as a white foam.

[1-[2',5'-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-(3-aminopropyl) thymine]-3'-spiro-5''-(4''-amino-1'',2''-oxathiole- 2'',2''-dioxide) (3)

A solution of compound 2 (1 equiv.) in methanol containing Pd/C (10%) (30 wt%) was hydrogenated at 25 psi at room temperature for 2 h. The reaction mixture was filtered, and the filtrate was evaporated to dryness, under reduced pressure to give compound 3 (90 %).

General procedure for the synthesis of TSAO-peptides-Z protected (4)

A solution of the corresponding peptide (1.5 equiv.) (prepared following usual coupling method in peptide synthesis) in dichloromethane (2 mL), was successively treated, at room temperature, with (benzotriazol-1-yl-oxy)-*tris*-(dimethylamino)-phosphonium hexafluorophosphate (BOP) (1.5 equiv.), amino-TSAO derivative 3 (1

equiv.) and triethylamine (1.5 equiv.). The reaction mixture was stirred until complete disappearance of the starting compound (3) (10-12 hours). Then, the solvent was evaporated to dryness and the residue was dissolved in dry dichloromethane (2 mL), washed with 10% aqueous citric acid (10 mL), 10% aqueous $NaHCO_3$ (10 mL) and brine (2 x 10 mL). The organic layer was dried (Na_2SO_4) and evaporated to dryness. The residue was purified by CCTLC on the Chromatotron using dichloromethane:methanol (70:1) as the eluent to give TSAO-dipeptides (4) (50-55 % yield)

General procedure for the synthesis of deprotected TSAO-peptides (5)

A solution of the corresponding TSAO-peptides-Z-protected (4) (1 equiv.) in methanol containing Pd/C (10%) (40% wt/wt) was hydrogenated at 25 psi at room temperature for 2 h. The reaction mixture was filtered, and the filtrate was evaporated to dryness, under reduced pressure to give 5 (90% yield) as a foam.

General peptide chemistry

Coupling of natural amino acids in order to form a peptide is straightforward for a person skilled in the art. Several chemical strategies are available of which the Fmoc and Boc chemistry are the most widely used. Fields G.B. gives an extensive description of the peptide chemistry that can be applied to couple amino acids to each other or to a therapeutic drug.(19) Solid phase as well as solution phase chemistry can be applied.(20)

Evaluation of the inhibitory effect of CD26 inhibitors on the conversion of TSAO-NAP-Val-Pro to TSAO-NAP by purified CD26, human serum and bovine serum

All enzyme activity assays were performed in Eppendorf tubes on a heating block at 37°C. To each tube were added 32 µl CD26 (at a final concentration of 1.5 milliUnits) or 10 µl foetal bovine serum (BS) (final concentration: 2.5% BS in PBS; preheated at 56°C for 30 min) or 10 µl human serum (HS) (final concentration: 2.5% HS in PBS), 40 µl of appropriate concentrations of inhibitor (Diprotin A or IlePyr) solution in PBS (for the exact concentrations, see the legend to Fig. 5), CAM-405 (the substrate of the reaction) at 50 µM (final concentration) and PBS to reach a total volume of 400 µl. The pH of the reaction mixture was 7.5, which is virtually identical to the physiological pH of plasma. The reaction was started by the addition of the enzyme or

serum and carried out at 37°C. After 5 hr, 100 µl reaction mixture was taken from the Eppendorf tube and added to 200 µl cold methanol to precipitate the proteins. After 10 min standing on ice, the contents of the tubes were centrifuged and the supernatants analysed by HPLC on a reverse phase column (RP-8, Merck Laboratories). CAM-405 was separated from CAM-212 (the product of the reaction) by a gradient of 50 mM sodium phosphate + 5 mM heptane sulfonic acid pH 3.2 (Buffer A) and acetonitrile (Buffer B) as follows: Buffer A: 98% + 2% Buffer B, 2 min; linear gradient to 20% Buffer B, from 2 to 8 min; linear gradient to 25% Buffer B from 8 to 10 min; linear gradient to 35% Buffer B from 10 to 12 min; linear gradient to 50% Buffer B from 12 to 30 min; 50% Buffer B from 30 to 35 min; linear gradient to 98% Buffer A + 2% Buffer B from 35 to 40 min; 98% Buffer A from 40 to 45 min. The retention times of CAM-405 and CAM-212 were 29.3 and 30.0 min, respectively.

Measuring the solubility and bioavailability of the prodrugs

In first instance methods exist to predict the solubility of a compound. For example in J Chem Inf Comput Sci 1998 May-Jun;38(3):450-6 the aqueous solubility prediction of drugs based on molecular topology and neural network modeling has been described.

In fact, all parameters relevant for solubility and bioavalability (pKa, partition coefficient, etc.) can be determined. "Drug Bioavailability: Estimation of Solubility, Permeability, Absorption and Bioavailability" gives a comprehensive overview of these parameters and their determination or prediction (ISBN 352730438X).

Partition coefficients are a measurement of lipophilicity. Expressed numerically as 'log P' values, they are the ratios between the concentrations of substances in two immiscible phases, such as water/octanol or water/liposomes and they can be easily calculated. Substances with high log P values dissolve better in fats and oils than in water. This enhances their ability to enter lipid (fat-based) membranes in the body by passive diffusion, thereby enhancing their potential for absorption.

Many drugs have a log P value of between one and four, making them suitable for oral methods of delivery. Drugs with high log P are usually poorly soluble in water. They may be lipid-soluble, but they cannot dissolve in the GI tract, so can't diffuse into the gut wall. If they do enter membranes, they may become trapped, with resultant toxic effects.

The partition coefficient can also be calculated. A method for logP prediction developed at Molinspiration (miLogP1.2) is based on the group contributions. Group contributions have been obtained by fitting calculated logP with experimental logP for a training set of several thousands drug-like molecules. The method can be used by used at www.molinspiration.com/services/logp.html (QSAR 15, 403 (1996)). Many other LogP determination programs are available.

REFERENCES TO THE APPLICATION

1. Han, H., de Vrueh, R.L., Rhie, J.K., Covitz, K.M., Smith, P.L., Lee, C.P., Oh, D.M., Sadee, W. & Amidon, G.L. Pharm. Res. 1998, 15: 1154-1159.

2. Han H.K., Oh, D.M. & Amidon, G.L. Pharm. Res. 1998, 15: 1382-1386.

3. Friedrichsen, G.M., Chen, W., Begtrup, M., Lee, C.-P., Smith, P.L. & Borchardt, R.T. Eur. J. Pharm. Sci. 2002, 16: 1-13.

4. Abbott, C., Baker, E., Sutherland, G.R. & McCaughan, G. Immunogenetics 1994, 40: 331-338.

5. Hegen, M. In: Leukocyte Typing VI. Kishimoto, T., ed. Garland Publishing, 1997, pp. 478-481.

6a. De Meester, I., Korom, S., Van Damme, J. & Scharpé, S. Rev. Immunol. Today 1999, 20: 367-375.

6b. Mentlein, R. Regul. Pept. 1999, 85: 9-24.

7. Trugnan, G., Ait-Sliminate, T., David, F. et al. In: Cell-Surface Peptidases in Health and Disease. Kenny, A.J. & Boustead, C.M., eds. BIOS, 1997, pp. 203-217.

8. Gonzalez-Gronow, M., Shearin, T. & Pizzo, S. Fibrinolysis 1996, 10 (Suppl. 3): 32.

9. Löster, K., Zeilinger, K., Schuppan, D. & Reutter, W. Biochem. Biophys. Res. Commun. 1995, 217: 341-348.

10. Ahmad, S., Wang, L. & Ward, P. Pharmacol. Exp. Ther. 1992, 260: 1257-1261.

11. Bouras, M., Huneau, J., Luengo, C., Erlanson-Albertsson, C. & Tomé, D. Peptides 1995, 16: 399-405.

12. Oravecz, T., Pall, M., Roderiquez, G., Gorrell, M.D., Ditto, M., Nguyen, N.Y., Boykins, R., Unsworth, E. & Norcross, M.A. J. Exp. Med. 1997, 186: 1865-1872.

13. Proost, P., De Meester, I, Schols, D., Struyf, S., Lambeir, A.M., Wuyts, A., Opdenakker, G., De Clercq, E., Scharpé, S. & Van Damme, J. J. Biol. Chem. 1998, 273:7222-7227.

14. Ohtsuki, T., Hosono, O., Kobayashi, H., Munakata, Y., Souta, A., Shioda, T. & Morimoto, C. FEBS Lett. 1998, 431: 236-240.

15. Proost, P., Struyf, S., Schols, D., Durinx, C., Wuyts, A., Lenaerts, J.P., De Clercq, E., De Meester I. & Van Damme, J. FEBS Lett. 1998, 432: 73-76.

16. Proost, P., Struyf, S., Schols, D., Opdenakker, G., Sozzani, S., Allavena, P., Mantovani, A., Augustyns, K., Bal, G., Haemers, A., Lambeir, A.M., Scharpé, S., Van Damme, J. & De Meester, I. J. Biol. Chem. 1999, 274: 3988-3993.

17. Senten, K., Van der Veken, P., Bal, G., De Meester, I., Lambeir, A.-M., Scharpé, S., Bauvois, B., Haemers, A. & Augustyns, K. Bioorg. Med. Chem. Lett. 2002, 12: 2825-2828.

18. De Meester, I., Vanhoof, G., Lambeir, A.-M. & Scharpé, S. J. Immunol. Methods 1996, 189: 99-105.

19. Fields, G.B. *Methods in Molecular Biology, Vol. 35: Peptide Synthesis Protocols* Humana Press Inc.: Totawa, 1994; 17 – 27.

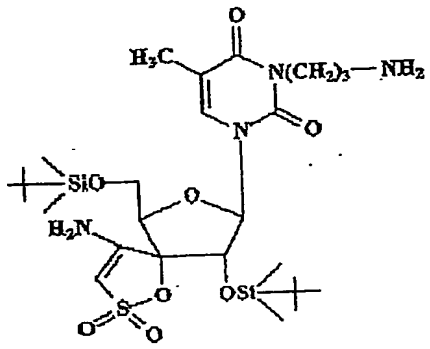
20. Atherton, A.; Sheppard, R.C. *Solid Phase Peptide Synthesis* IRL Press: Oxford-New York-Tokyo, 1989.

CLAIMS

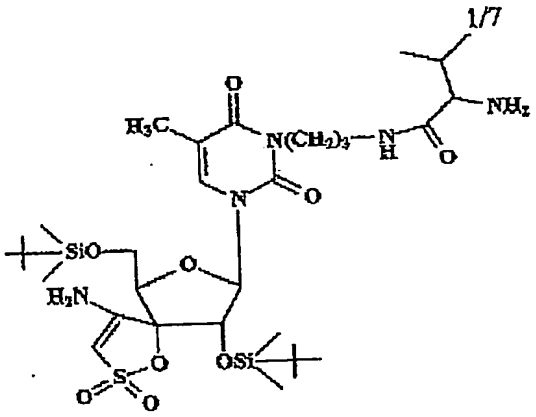
1. A prodrug, characterised in that the prodrug is cleavable by a dipeptidyl-peptidase.
2. A prodrug according to claim 1, wherein the prodrug is a conjugate of a therapeutic drug and a peptide and derivatives thereof.
3. A prodrug according to claim 2, wherein the therapeutic drug and the peptide are coupled through an amide bond.
4. A Prodrug according to claim 3, wherein the therapeutic drug and an the peptide are indirectly coupled via a linker.
5. A prodrug according to claims 2 to 4, wherein said peptide comprises $(X\text{-}Y)_n$ repeated units wherein X represents any amino acid, Y is selected from Pro, Ala, Gly, Ser, Val or Leu and n is selected from 1, 2, 3, 4 or 5.
6. A prodrug according to claims 2 to 4, wherein the peptide ends aminoterminally with X-Pro or X-Ala.
7. A prodrug according to claims 5 and 6, wherein X is selected from the hydrophobic amino acids (i.e. Val, Leu, Ile, Phe and Tyr).
8. A prodrug according to claims 2 to 7, wherein the peptide is a dipeptide.
9. A prodrug according to all previous claims, wherein the dipeptidyl-peptidase is dipeptidyl peptidase IV (EC 3.4.14.5 - CD26).
10. A prodrug according to all previous claims, wherein the therapeutic drug is an anti-viral drug.
11. A prodrug according to claim 10, wherein the antiviral drug is TSAO.
12. A method for increasing the water solubility, decreasing plasma protein binding and/or the bioavailability of a therapeutic drug by coupling a peptide to said therapeutic drug whereby the resulting conjugate is cleavable by a dipeptidyl-peptidase.
13. A method of producing a prodrug, wherein the prodrug is cleavable by a dipeptidyl-peptidase, the method comprising the step of linking a therapeutically active drug and a peptide whereby the resulting conjugate is cleavable by dipeptidyl-peptidases.
14. A method of selecting potential prodrugs, said method comprising contacting amino acid prodrugs with dipeptidyl-peptidases or tissue or cells producing dipeptidyl-peptidases and with dipeptidyl-peptidases free medium in a parallel experiment.

ABSTRACT

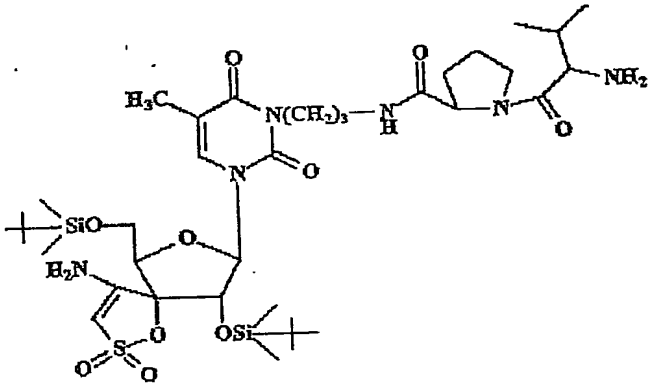
The present invention provides a new prodrug technology and new prodrugs in order to increase the solubility, to decrease plasma protein binding or to enhance the bioavailability of a drug. In the present invention the prodrugs are conjugates of a therapeutic drug and a peptide wherein the conjugate is cleavable by dipeptidyl-peptidases, more preferrably by dipeptidyl aminodipeptidase IV. The present invention furthermore provides a method of producing said prodrugs. The invention also provides a prodrug technology to more selectively target drugs, to enhance brain and lymphatic delivery of drugs and/or to extend drug half-lives in plasma.



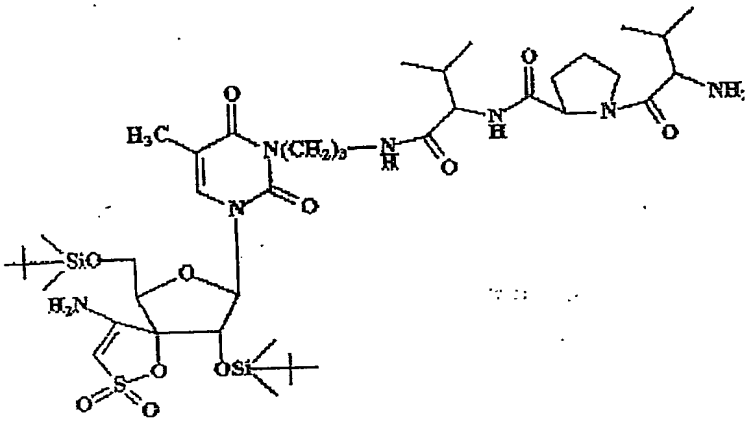
TSAO-NAP
(CAM-212)



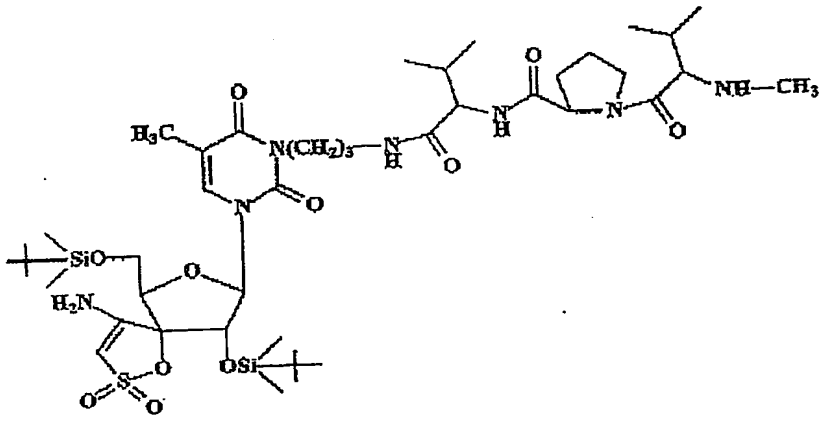
TSAO-NAP-Val
(CAM-403)



TSAO-NAP-Pro-Val
(CAM-405)



TSAO-NAP-Val-Pro-Val
(CAM-431)



TSAO-NAP-Val-Pro-Val-CH_3
(CAM-407)

Fig. 1

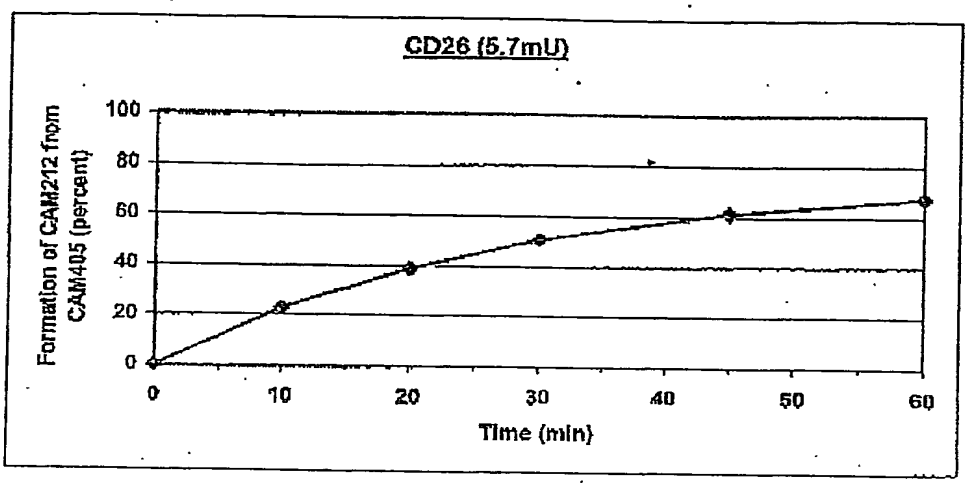

CD26 (5.7mU)
Formation of CAM212 from CAM405 (percent)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (min)

Fig.2

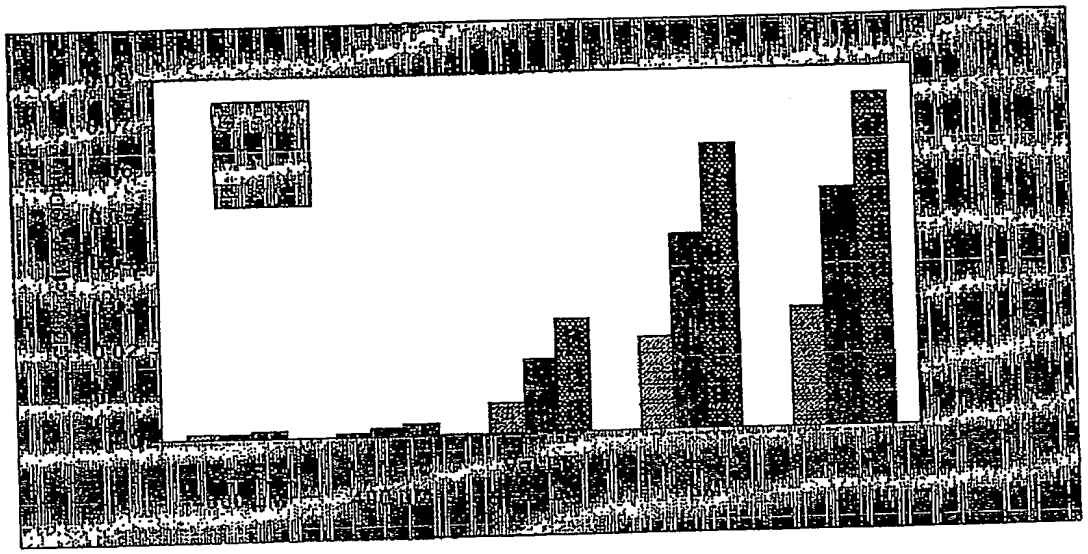


Fig. 3

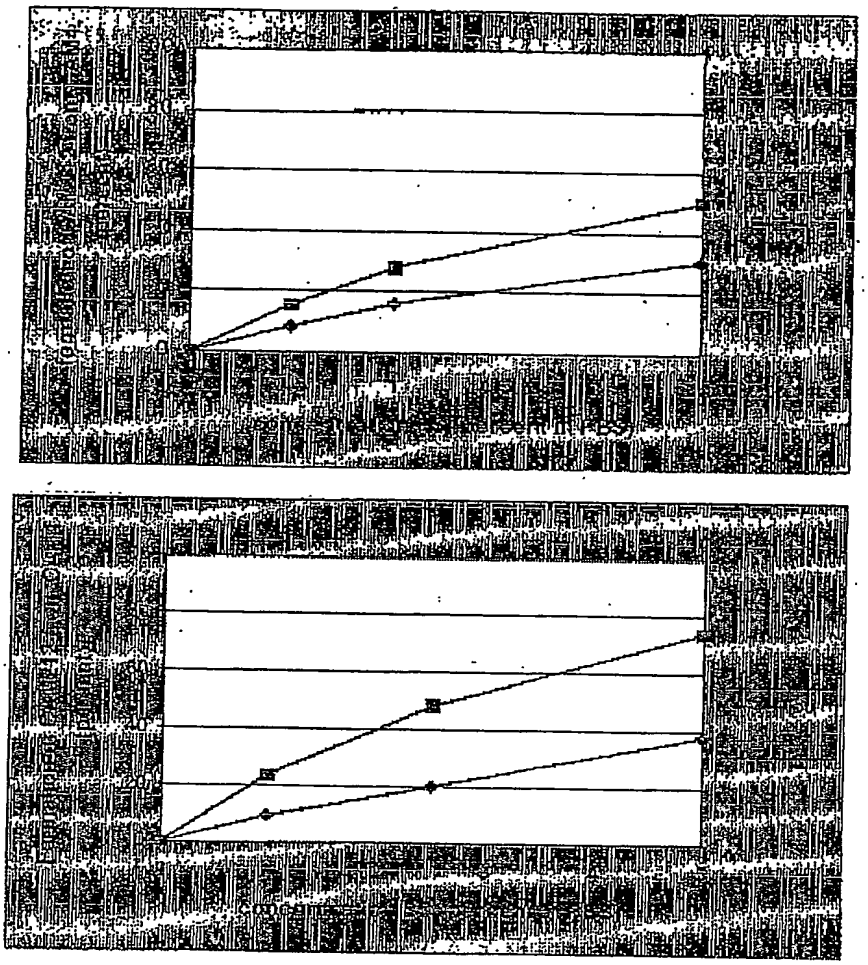
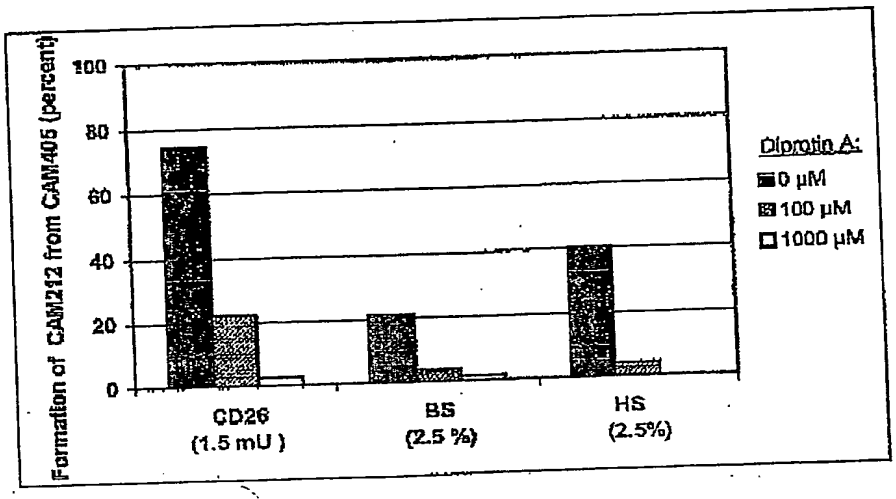
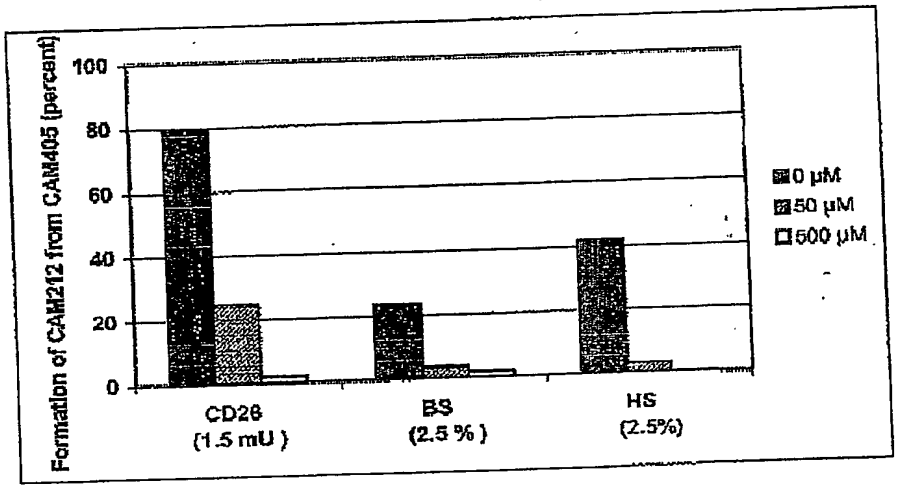


Fig. 4


Formation of CAM212 from CAM405 (percent)
100
80
60
40
20
0
CD26 (1.5 mU)
BS (2.5 %)
HS (2.5%)
Diprotin A:
0 µM
100 µM
1000 µM


Formation of CAM212 from CAM405 (percent)
100
80
60
40
20
0
CD26 (1.5 mU)
BS (2.5 %)
HS (2.5%)
0 µM
50 µM
500 µM

B

Fig. 5

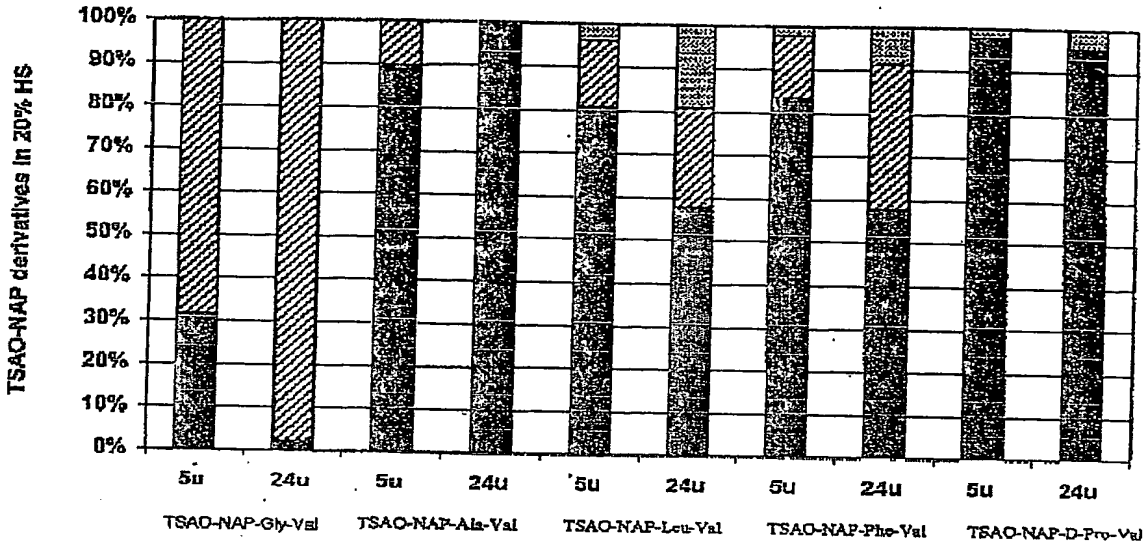

TSAO-NAP derivatives in 20% HS
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
5u
24u
TSAO-NAP-Gly-Val
TSAO-NAP-Ala-Val
TSAO-NAP-Leu-Val
TSAO-NAP-Phe-Val
TSAO-NAP-D-Pro-Val

Fig. 6

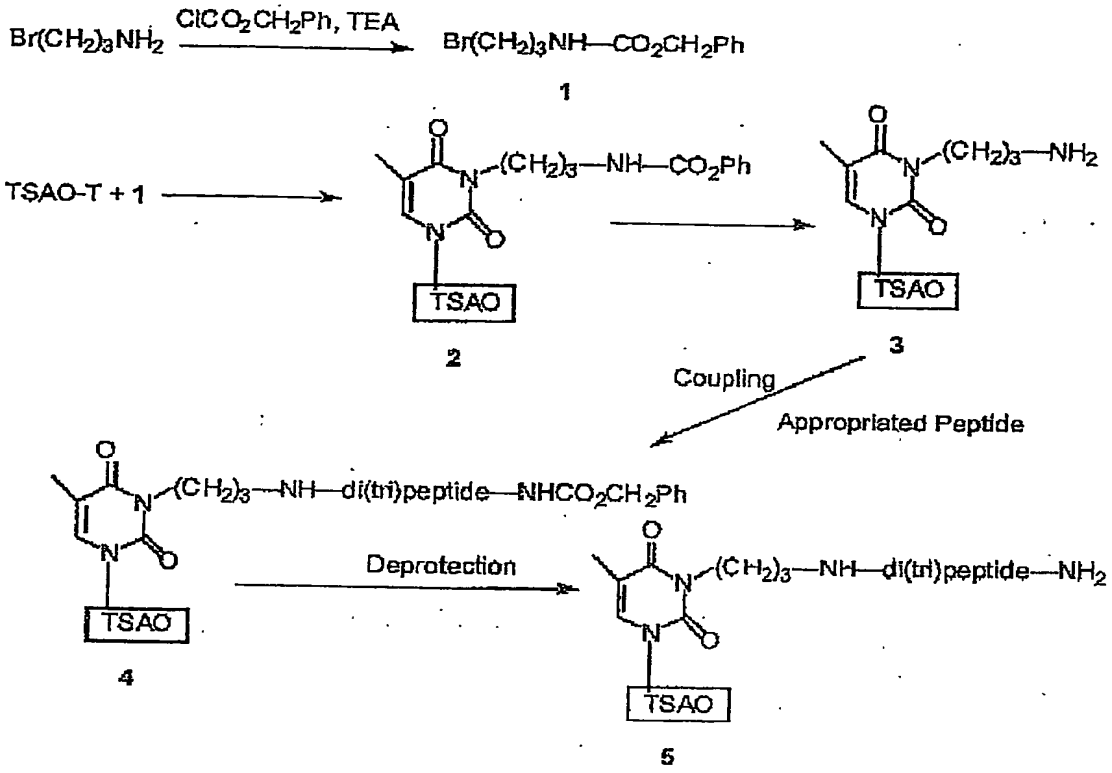

Br(CH2)3NH2
ClCO2CH2Ph, TEA
Br(CH2)3NH—CO2CH2Ph
1
TSAO-T + 1
(CH2)3—NH—CO2Ph
TSAO
2
(CH2)3—NH2
TSAO
3
Coupling
Appropriated Peptide
(CH2)3—NH—di(tri)peptide—NHCO2CH2Ph
TSAO
4
Deprotection
(CH2)3—NH—di(tri)peptide—NH2
TSAO
5

Fig. 7